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<p>(54) Title: DNA FOR DIAGNOSING PNEUMOCYSTIS CARINII</p> <p style="text-align: center;">         5' - G A T G G C T G T T T C C A A G C C C A - 3'          5' - G T G T A C G T T G C A A A G T A C T C - 3'          (I)       </p> <p>(57) Abstract</p> <p>A method of assaying a sample of DNA from respiratory secretion of a patient for <i>Pneumocystis carinii</i>, comprises amplifying a polynucleotide sequence derived from <i>P. carinii</i> by a polymerase chain reaction, and detecting the amplified sequence if present. Two DNA sequences are given, together with a number of pairs of oligonucleotide primers, including particularly the pair (I).</p>			

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DNA FOR DIAGNOSING PNEUMOCYSTIS CARINIIIntroduction

5        Pneumocystis carinii is established as the prime cause of opportunistic pneumonia in patients with AIDS and those immunosuppressed on oncology and transplant units. Debate over the taxonomy of P.carinii continues and the fastidious nature of the 10. parasite still demands the use of microscopy after histochemical staining or immunofluorescence for the detection of the parasite in diverse forms of lung samplings [1,2]. The requirement for observer expertise is significant and limits the diagnostic 15 power of these techniques. There are sound theoretical grounds for believing that DNA amplification using the polymerase chain reaction (PCR) [3] might provide both a specific and sensitive means of identifying the parasite in clinical samplings and one that finally 20 might be amenable to automation. Other applications of DNA amplification to the study of the epidemiology of P.carinii infection are also evident.

European Patent Specification 327390 describes DNA sequences produced by recombinant DNA 25 technology from an experimental rat model which hybridise to DNA of P.carinii but not to mammalian DNA. These DNA sequences were present as inserts in plasmids of which two were designated pAZ 102 and pAZ 112. This invention results from further work described herein:- 30 A.        We have sequenced the inserts of pAZ 102 and pAZ 112. The sequences are set out in Figures 1 and 3. B.        Using suitable oligonucleotide primers we have amplified, by the polymerase chain reaction (PCR) technique, P.carinii DNA from infected rat lung 35 samplings, to an extent that the amplified DNA was

easily detectable by staining an electrophoresis gel. In the same way, we have amplified P.carinii DNA from infected human lung samplings.

C. Analysis of the amplified DNA from human samples has shown significant sequence differences from 5 the infected rat material. The human sequence is set out in Figure 1, and the similarities and differences between human and rat sequences are highlighted.

D. The human sequence, leads to improved 10 oligonucleotide primers which more efficiently amplify P.carinii DNA of human origin.

E. We have developed a more sensitive detection system, involving hybridising the amplified DNA to a labelled probe which probe is part of the sequence 15 determined in C. intermediate the two primers. By these means we are able to detect P.carinii DNA, and thus to diagnose infection by P.carinii, in patients who do not (yet) show clinical symptoms.

In one aspect, this invention provides, as 20 new chemical compounds, the nucleic acid sequences shown in Figures 1 and 3, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at 25 least 90% homology thereto. These result from steps A. and C. above.

25 In another aspect, the invention provides a method of assaying a sample of DNA from respiratory secretion of a patient possibly infected with Pneumocystis carinii, which method comprises using a 30 pair of oligonucleotide primers based on the sequences shown in Figure 1 or Figure 3 to amplify by a polymerase chain reaction a polynucleotide sequence derived from P.carinii if such sequence is present in the sample, and detecting the amplified sequence if 35 present. This is step B. above, and steps D. and E. constitute preferred features of the method.

For maximum efficiency and specificity of the PCR reaction, the choice of oligonucleotide primers is critical. The primers must be based on the sequence to be amplified and may be identical to the two ends.

5 However, identity is by no means essential (R. Sommer and D. Tautz, Nucleic Acids Research, Vol. 17, No. 16, 1989, p. 6749). Generally the two or three nucleotides at the 3'-end of the primer, and at least 50% (preferably at least 90%) of all the nucleotides of the primer, are homologous to the sequence to be amplified.

10 The primers are partly or completely homologous to particular sites of the sequence to be amplified. For maximum efficiency of the PCR reaction, the location of those sites is also important.

15 Although most primers will work with varying degrees of success, general guidelines for obtaining useful primers are found in the literature (see Saiki R. K. et al., The Polymerase Chain reaction in Genome Analysis (Ed K. E. Davies) IRL, Oxford, 1988). However, the design of effective primers tends to be empirical.

20 Described below are one pair of primers derived from pAZ 102 that have proved outstanding; and several pairs of primers derived from pAZ 112 that have proved effective.

25 In other respects, the PCR conditions may be conventional. The primers may be at least 8, conveniently about 20, nucleotides in length. The number of cycles required to achieve sufficient amplification may be from 15 to 50. If required to improve specificity, two different pairs of primers may 30 be used. The resulting amplified sequence has a predetermined length, and moves a predetermined distance on an electrophoresis gel. The resulting band can be visualised, either by conventional staining techniques, or by hybridisation to a labelled probe 35 which probe is homologous to part or all of the known

sequence being amplified.

Reference is directed to the accompanying Figures, in which:-

Figure 1 comprises sequence data on different DNA samples. Row 1 entitled "Rat" is from 5 lung samplings from a rat infected with P. carinii. Row 2 entitled "Human" is from lung samplings of infected humans. Secondary structure has been taken into consideration and gaps (-) introduced to obtain maximum alignment. Numerous differences between human 10 and rat sequences are shown boxed.

Figure 2 is a diagram of the circular plasmid pAZ 112 showing certain features including the positions of polynucleotide primers used in PCR.

Figure 3 comprises the complete sequence of 15 the insert of pAZ 112, with the oligonucleotide primers marked. R/C means reversed and complemented, i.e. the actual sequence of the primer is the reverse and complementary to that marked.

Table 2 lists the oligonucleotide primers 20 referred to in Figures 2 and 3.

Table 3 lists the primer combinations successfully used by us and the approximate size of the resulting amplification product.

The following Examples illustrate the 25 invention. Example 1 relates to DNA from the plasmid pAZ 102 whose sequence is shown in Figure 1. Example 2 relates to DNA from the plasmid pAZ 112 whose sequence is shown in Figure 3. Example 3 reports a clinical trial following the method of Example 1.

30

#### Example 1

#### Methods

cloning and sequencing of part of the gene coding for the large sub-unit of the mitochondrial ribosomal RNA from *P. carinii*

*P. carinii* pneumonia was induced in the rat model and DNA extracted and cloned from a parasite enriched fraction as previously described [4]. *P. carinii* specific sequences were confirmed by characteristic in situ hybridisation patterns and recombinant plasmid pAZ102 was selected as a candidate mitochondrial sequence because of strong signals derived in dot blot hybridisation studies on infected samples. The recombinant plasmid pAZ102 (insert 570 bp) was sequenced using Sanger's chain termination method and the Sequenase kit (United States Biochemical Corporation, Cleveland, USA), <sup>35</sup>S (Amersham, UK), Sequagel (National Diagnostics, Manville, USA). The DNA sequence was compared with those available in several databases including EMBL and Genbank. From the sequence data on pAZ102 and comparative analysis of the databases, the fragment was identified as a portion of the gene coding for the large sub-unit of the mitochondrial, ribosomal RNA of *P. carinii* and this showed significant homology with fungal sequences (manuscript in preparation).

oligonucleotide primers

Two sequences of moderate conservation that were specific to *P. carinii* were selected for construction of oligonucleotide primers for the polymerase chain reaction:- pAZ102 - E:-5'-GATGGCTGTTCCAAGCCCA-3'; pAZ102-H:-5'-GTGTACGTTGCAAAGTACTC-3'. An oligonucleotide for confirmatory Southern hybridisation on amplification products was chosen, pAZ102-L1. Subsequently a new internal oligonucleotide specific to human *P. carinii* sequences was constructed, pAZ102-L2 (Table 1).

Template DNA

- i) Samplings for DNA amplification using our oligonucleotide primers comprised a) pulmonary lavage samplings from 3 humans and 3 rats with *P. carinii* pneumonia documented by methenamine silver staining and microscopy, and b) isolates from a series of organisms including some

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potential pulmonary pathogens: Candida (an albicans and a non-albicans strain), Cryptococcus neoformans, Mycobacterium tuberculosis, Saccharomyces cerevisiae and Aspergillus nidulans.

ii) Template DNA was prepared from each sample by proteinase K digestion in the presence of SDS and EDTA, followed by phenol/chloroform/ether extraction, and ethanol precipitation.

DNA amplification

Using primers pAZ102-E and pAZ102-H the template samples, together with control samples without template underwent 40 cycles of amplification performed with denaturation at 94°C for 90 seconds, annealing at 50°C for 90 seconds and extension at 72°C for two minutes (Techne, UK). The DNA amplification reaction mixture (50 µl) contained 50mM KCl, 10 mM Tris, pH 8, 0.01% (w/v) gelatin, 3mM MgCl<sub>2</sub>, 400 µM dNTPs (Boehringer Mannheim, UK), 0.4-1.0 µM oligonucleotide primer and 3 units of AmpliTaq (Perkin Elmer Cetus, UK).

To avoid the possibility of false negative results in the human clinical samples, i.e. failure to detect the specific amplification product for technical reasons, we carried out a parallel polymerase chain reaction on each sample using primers derived from the human anti-thrombin gene, exon 2

These primers, (AT1: -5'-

GTTGCAGCCTAGCTTAACTTGGCA-3'; AT4: -5'-GGTTGAGGAATCATTGGACTTG-3') allowed amplification to take place using human genomic DNA as template. In each of the clinical samples the 500 bp specific product was detected, demonstrating efficient amplification.

The potential problem of contamination in PCR was monitored by systematic use of the following techniques: i) including several negative control samples with no added template DNA; ii) by the use of UV-irradiation of the PCR reaction mixes prior to the addition of the template DNA [5]; iii) the use of separate disposable microcapillaries for the addition of each template (Laser Laboratory Systems Ltd, UK). The control

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samples remained negative in all experiments.

Amplified products (10  $\mu$ l) were electrophoresed in 1.5% agarose gels and visualised after ethidium bromide staining by ultraviolet light. The gel was Southern blotted on to Hybond N (Amersham, UK) and hybridised with  $^{32}$ P end-labelled internal primer at 46°C (pAZ102-L1) or 40°C (pAZ102-L2) for 3 hours[6]. Filters were subsequently washed at high stringency at 54°C (pAZ102-L1) or 48°C (pAZ102-L2) and filters exposed to radiographic film at -80°C with intensifying screens. The expected amplification product was 355bp long in the rat derived parasite, that from the human derived parasite being 9 bp shorter.

#### Sequencing of products of DNA amplification

The PCR product was gel purified and recovered from the agarose gel using Geneclean (Bio 101, Inc). The purified DNA was heat denatured and sequenced as described above using primers pAZ102-H or pAZ102-E at 20pmole/ $\mu$ l.

#### Results

The oligonucleotide primers derived from rat *P.carinii* produced efficient amplification of specific sequence from both rat and human hosts, shown by ethidium bromide staining but none from the range of other organisms including some potential pulmonary pathogens.

The internal oligonucleotide, pAZ102-L1, derived from the rat *P.carinii*, produced strong hybridisation signals on Southern hybridisation with amplified products from the infected rat lungs, but weak signal, at high stringency, with the amplified product derived from human samples although these were visible on ethidium bromide staining. Direct sequencing of the amplified products from each of the rat and human samples allowed comparison of their sequence and demonstrated limited but consistent differences between the *P.carinii* DNA from these two hosts which included 5 base changes in the sequence of the internal oligonucleotide pAZ102-L1 (Table 1).

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An oligonucleotide specific to the human derived organisms was constructed, pAZ102-L2, which showed strong hybridisation with the amplified product from human P.carinii and conversely showed weak hybridisation with the rat P.carinii amplified product. It produced no hybridisation with the PCR products of the range of other organisms tested.

Studies using serial dilutions of human derived P.carinii template DNA indicated that the application of oligoblotting with pAZ102-L2 to amplified DNA products increased the sensitivity of detection by at least 100 fold over visualisation by ethidium bromide staining.

The P.carinii oligonucleotide primers successfully amplified specific PCR product from bronchoscopic alveolar lavage samplings from 10 HIV-positive individuals with pneumocystis pneumonia as documented by positive methenamine silver staining on the lavage samples. Lavage samples from 5 immunocompetent subjects were studied as controls. These failed to show specific PCR product by ethidium bromide staining or oligoblotting.

#### Discussion

We have characterised a portion of the gene coding for the large sub-unit of mitochondrial, ribosomal RNA from P.carinii and comparative analysis of this indicates significant homology with fungal sequences. This result accords with data we have on other mitochondrial genes and the observations of other groups on ribosomal RNA[7].

We have identified P.carinii specific sequences from which we have constructed oligonucleotide primers which allow efficient amplification of part of this ribosomal RNA gene from P.carinii infecting both rat and human hosts but not from a range of other organisms including some potential pulmonary pathogens. These results indicate the specificity of the amplified products to P.carinii, confirmed on Southern hybridisation with internal oligonucleotide pAZ102-L1 derived from the rat sequence and applied to the rat pulmonary samplings. Results from the human samplings suggested the likelihood of differences in sequence between the amplified products from the rat and human. This was confirmed by comparison of sequences which indicated limited, but consistent differences.

This finding is highly relevant to the hopes for developing DNA amplification as a diagnostic tool in clinical medicine. We have shown that our oligonucleotide primers can be used to identify the presence of P.carinii in a number of bronchoalveolar lavage samples. By using our second internal oligonucleotide, pAZ102-L2, which is specific to human P.carinii, the sensitivity of detection of amplified product is considerably increased. This method shows great potential for use on non-invasive samples such as induced sputum where parasite numbers are lower. It will not only be valuable in diagnosis but also in addressing questions relevant to the epidemiology of P.carinii.

The application of DNA amplification to diagnosis will require careful calibration to ensure that levels of P.carinii in keeping with clinical pneumonia can be distinguished from lesser degrees of colonisation that are likely to occur in the immunodeficient before clinical disease is manifest. Such methods of calibration of DNA amplification are becoming available [8,9] and their application to diagnostic studies on P.carinii in diverse clinical samplings, including lavage and induced sputum, are now required.

Example 2

For pAZ 112 we used the techniques described in Example 1, and the oligonucleotide primers given in Table 2 in the combination set out in Table 3. We 5 achieved amplification by PCR of the sequences of pAZ 112 shown in Figure 3.

Example 310 Clinical Specimens

Alveolar lavage samples were obtained from 47 patients investigated by bronchoscopy at the Churchill Hospital, Oxford, and at the Middlesex Hospital, 15 London.

Thirty seven patients were immunosuppressed, either by HIV infection (33) or by treatment for lymphoma (2), vasculitis (1) or leukaemia (1). All 20 patients had symptoms of acute respiratory illness with one or more of the following features: abnormal chest signs, arterial hypoxaemia, or abnormal chest radiograph. The 10 remaining patients were immunocompetent, undergoing bronchoscopy to investigate 25 various respiratory disorders. Routine microbiological and cytological analysis including methenamine silver staining was performed on each lavage and an aliquot reserved for the DNA amplification study, performed as described in Example 1.

30 Results

On the basis of clinical progress, response to treatment with nebulised pentamidine (20 cases) or cotrimoxazole (7 cases) and results of standard 35 investigation including methenamine silver staining,

the 47 patients were eventually categorised into four groups (Table 4); 16 immunosuppressed patients with a positive diagnosis of pneumocystis pneumonia by silver staining on lavage and response to treatment, 6 immunosuppressed patients with clinical response to treatment, but negative silver stains on lavage, 15 immunosuppressed patients with neither response to treatment nor positive silver staining on lavage and in whom an alternative diagnosis to account for the respiratory disease was available in 12, and the 10 immunocompetent patients from the routine bronchoscopy list. The results of DNA amplification assayed by the visualisation of a 346 base pair DNA band after a) ethidium bromide staining and b) autoradiography after oligoblotting were compared with these clinical data categorisations (Table 4).

No P.carinii DNA was detectable in the samples from the immunocompetent group. All of the 16 immunosuppressed individuals with P.carinii identified by methenamine silver staining on alveolar lavage had amplified P.carinii DNA visible by both ethidium bromide staining and oligoblotting. Of the 6 individuals judged to have had pneumocystis pneumonia by clinical symptoms and response to treatment but not confirmed by identification of parasites in methenamine silver stained lavage samples, 4 were positive using DNA amplification - both by ethidium bromide staining and oligoblotting - and 2 negative by both methods.

Lesser degree of P.carinii infection were detected in the oligoblots - but not by ethidium bromide staining - of the DNA amplification product in lavage samples from 3/15 of the immunosuppressed subjects without pneumocystis pneumonia. The intensity of the signal was less than that obtained from patients with acute P.carinii infection but significantly greater than a barely visible signal obtained in 4

other samples from this patient group, and from 3 of 12 preliminary washings of the bronchoscope after routine cleaning and sterilisation.

Example 4

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The method of Example 1 was extended to test induced sputum samples. Fifty one episodes of acute respiratory illness in immunosuppressed HIV-infected individuals were studied. Bronchoscopic alveolar lavage was obtained from each patient and in thirty seven instances induced sputum was also obtained. Samples were examined by routine microbiological and cytological methods, including methenamine silver staining for P. carinii; a part of each sample was reserved for DNA amplification. DNA was extracted from 1 ml lavage or sputum by proteinase K digestion (1 mg/ml final concentration of proteinase K, in the presence of 10 mmol EDTA, pH 8.0 and 1% weight/volume sodiumdodecylsulphate, at 50°C for 16 hours and phenol/chloroform extraction. DNA amplification was done with the oligonucleotide primers pAZ102-E and pAZ102-H, with denaturation at 94°C for 90 s, annealing at 55°C for 90 s, and extension at 72°C for 2 min (40 cycles). The amplification products were subjected to electrophoresis in 1.5% agarose gel and the specific P. carinii sequence (346 base pairs) was identified by visualisation with ultraviolet light after ethidium bromide staining or by oligohybridisation, after Southern transfer and autoradiography with the internal primer pAZ102-L2. Scoring of the DNA bands was done without knowledge of the results of silver staining or of final clinical diagnosis, which was assessed by clinical features and response to treatment with co-trimoxazole or pentamidine.

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The results from the paired lavage and sputum

samples are summarised in the table 5 below. All 14 patients who had a final clinical diagnosis of pneumocystis pneumonia and who also had a positive silver stain on lavage, had a strong signal of amplified pneumocystis DNA from both the lavage sample and sputum. In 5 other patients with a final clinical diagnosis of pneumocystis pneumonia but negative silver stains, 4 were strongly positive by DNA amplification in alveolar lavage; 3 of these 4 were also positive in induced sputum. Silver stain was positive in only one third of sputum samples from cases of pneumocystis pneumonia.

Table 5

15	Final clinical diagnosis (numbers)	DNA amplification positive sputum	DNA amplification positive lavage	silver stain positive sputum	silver stain lavage
20	Pneumocystis pneumonia (20)	18	19	7	14
25	Other diagnoses (17)	1	1	0	0

Positive signals of amplified DNA can be categorised as strong (visible after ethidium bromide staining of the agarose gel) or weak (visible only on autoradiography after oligoblotting). Independent calibration experiments have shown that a strong signal points to 100 organisms or more in a sample, whereas a weak signal indicates from 1-2 organisms up to 100 organisms per sample. In broad terms, it may be said that patients providing samples with strong signals show clinical symptoms of pneumocystis pneumonia.

whereas patients providing samples with weak signals are in the pre-clinical stage. Thus, in 20 cases judged to have clinical pneumocystis pneumonia, a strong DNA amplification signal was obtained in 19 (95%) of the lavage samples and in 18 (90%) of the 5 paired sputum samples. By contrast, microscopy after silver staining could only diagnose 35% of these cases on induced sputum. The sensitivity of the DNA method is therefore excellent; it is unlikely that the single 10 case, negative by both DNA amplification and silver staining on lavage, had pneumocystis pneumonia.

The specificity of DNA amplification may be judged from the results of another study involving 44 patients, in whom the final clinical diagnosis was of another respiratory illness (i.e. not pneumocystis 15 pneumonia). A strong amplification signal was obtained both in the lavage and sputum samples in only one of these 44 patients; this patient had had a previous episode of pneumocystis pneumonia and returned with a further documented episode within ten weeks of 20 the current study.

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Table 1

Comparative sequence of oligonucleotides pAZ102-L1 (rat P. carinii) and pAZ102-L2 (human P. carinii).

pAZ102-L1	5' -	A	T	A	A	G	G	T	G	A	G	G	A	G	T	C	G	A	G	A	G	- 3'
pAZ102-L2	5' -	A	T	A	A	G	G	T	A	G	A	T	A	G	T	C	G	A	A	A	G	- 3'

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Table 2

DAZ 112 - Oligos used in PCR

	Name	Sequence	Length mer
5	1F	A G A A C T G G A T T C T T A G A	17
	1R	A G A A G T A T C A A G T T G A T	17
10	9F	C T C A T G C T T C A A G T G A C	17
	3F	C A C T T T T A C T A A T A G C C	17
	3R	T T G A T T A T C C A A C C A A G A	18
15	4F	T A A A T C C A C A T T C A A A G	17
	4R	T G T T T T T A G T T A A C C C T	17
20	5F	T A C G G G A T T G A G A T A A T	17
	5R	T T T A T G A T G G A G T A C C A	17
	6F	T A T T T G G A A T T G G A T G A	17
25	6R	T C T T T G C C T T G T T A G G A	17
	10F	T A G A C G G T C A C A G A G A T C A G	20
30	10R	G A A C G A T T A C T A G C A A T T C C	20

Table 3

PCR Results for pAZ 112

<u>Oligos</u>	<u>Approx. Size of Amplification Product, bp</u>
5 1F + 9F	285
10 3F + 3R	516
15 4F + 4R	198
15 5F + 5R	183
15 6F + 6R	148
20 10F + 10R	700
25	
30	
35	

Table 4

Results of DNA amplification of *P. carinii* DNA in bronchoalveolar lavage samples: comparison with methenamine silver staining and clinical response to treatment

Patient group (Number of Patients)	Silver Stain	Response to treatment	Positive DNA amplification for <i>P. carinii</i>		Negative DNA amplification for <i>P. carinii</i>
			ethidium bromide stain + Oligoblot	Oligoblot alone	
Immunosuppressed (16)	+	+	16	0	0
			4	0	2
			0	3 †	12 †
Immunocompetent (10)	-	-			10
			0	0	10

† Alternative diagnosis: *Toxoplasma gondii*; *Mycobacterium avium intracellulare*; Pulmonary lymphoma

‡ Alternative diagnosis: Endobronchial Kaposi's sarcoma (2), *Mycobacterium avium intracellulare* and *intracellulare* (2), *Mycobacterium avium intracellulare* and *cytomegalovirus* (1), *Salmonella typhimurium* (1), *Pseudomonas putida* (1), *Streptococcus pneumoniae* and *Haemophilus influenzae* (1), bronchiectasis (1), no diagnosis (3)

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CLAIMS

5 1. The nucleic acid sequences shown in Figure 1,  
single and double chain fragments thereof at least 15  
nucleotides in length, and nucleic acid sequences and  
fragments having at least 90% homology thereto.

10 2. The nucleic acid sequence shown in Figure 3,  
single and double chain fragments thereof at least 15  
nucleotides in length, and nucleic acid sequences and  
fragments having at least 90% homology thereto.

15 3. Peptide sequences transcribed from the  
nucleic acid sequences claimed in Claim 1 or Claim 2.

15 4. A method of assaying a sample of DNA from  
respiratory secretion of a patient possibly infected  
with Pneumocystis carinii, which method comprises using  
a pair of oligonucleotide primers based on the  
sequences shown in Figure 1 or Figure 3 to amplify by a  
20 polymerase chain reaction a polynucleotide sequence  
derived from P. carinii if such sequence is present in  
the sample, and detecting the amplified sequence if  
present.

25 5. A method as claimed in Claim 4, wherein the  
amplified sequence is detected by electrophoresis and  
staining.

30 6. A method as claimed in Claim 4, wherein the  
amplified sequence is detected by hybridisation to a  
labelled probe which probe is a nucleic acid sequence  
according to Claim 1 or Claim 2.

7. A method as claimed in Claim 7, wherein the  
probe is the 20-mer.

5' - A T A A G G T A G A T A G T C G A A A G - 3'

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8. A method as claimed in any one of Claims 4 to 7, wherein the oligonucleotide primers are:-

5' - G A T G G C T G T T T C C A A G C C C A - 3'

5' - G T G T A C G T T G C A A A G T A C T C - 3'.

9. A method as claimed in any one of claims 4 to 8, wherein the respiratory secretion assayed is induced sputum.

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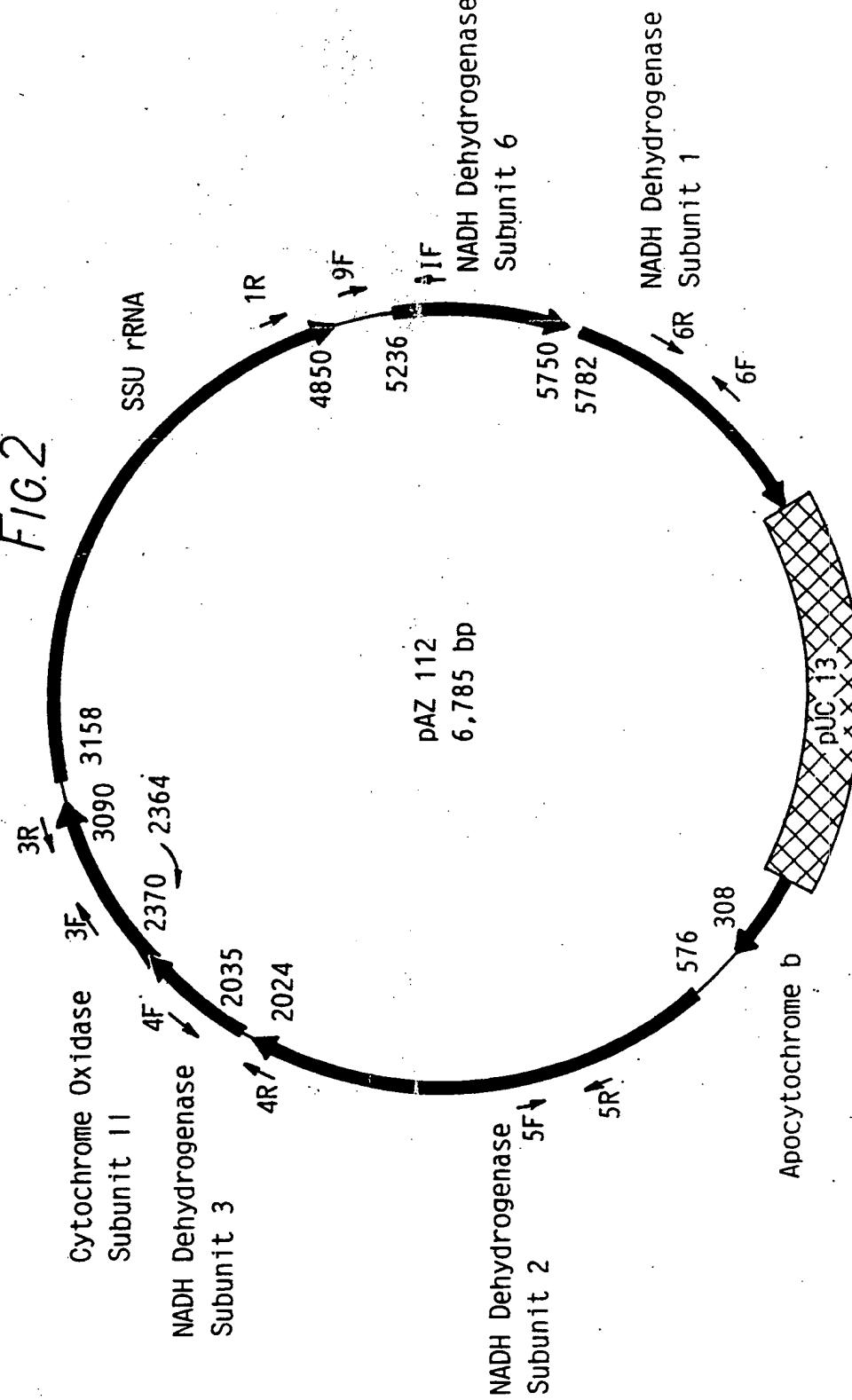
FIG. 1

AGAAG  
AGAAG

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FIG. 2



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FIG.3

D L F L I N C \* G L \* Q \* \* P Q Y \* Y C  
 I Y F \* \* I V R G C S N D S L / N I S I V  
S I S D K L L G V V A M I A S I L V L F  
 GATCTATTTCTGATAAAATTGTTAGGGGTTGTAGCAATGATAGCCTCAATATTAGTATTGT  
 10 20 30 40 50 60

S Y Y L S \* I Y L E F E D L S F D L \* V  
L I T S L R F I \* N S R I C L S T S K \*  
L L P L L D L S R I R G S V F R P L S K  
 TCTTATTACCTCTCTAGATTATCTAGAATTGAGGATCTGTCTTCGACCTCTAAGTA  
 70 80 90 100 110 120

N S S F G S L L Q I S Y Y \* C T \* D L N  
I L L L D L C Y K F L I I N V L R I S T  
F F F W I F V T N F L L L M Y L G S Q H  
 AATTCTTCTTTGGATCTTGTACAAATTCTTATTATTAAATGTACTTAGGATCTAAC  
 130 140 150 160 170 180

M \* K N L I L L L V D M E P Y F T S H I  
C R R T L Y Y Y W \* I W N P T L L L I F  
V E E P Y I T I G R Y G T L L Y F S Y F  
 ATGTAGAAGAACCTTATATTACTATTGGTAGATATGGAACCCCTACTTACTTCTCATATT  
 190 200 210 220 230 240

L S L \* Y Q L \* Q \* L R I L \* Q I \* L \*  
C L Y S T N Y S S D \* E Y F S R S S F N  
V F I V P I A V I E N T L A D L A L T  
 TTGTCTTATAGTACCAATTATAGCAGTGATTGAGAATCTTAGCAGATCTAGCTTAA  
 250 260 270 280 290 300

Q N N S G F L L F K K V F F L E S P I R  
K I I Q D F Y Y L K R Y S F W K V R Y E  
K \* F R I F I I \* K G I L F G K S D T S  
 CAAAATAATTAGGATTTTTATTATTAAAAAGGTATTCTTTGGAAAAGTCCGATACGA  
 310 320 330 340 350 360

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V K D N R I K S I L \* K \* K T F R R N \*  
 L K I I E \* N L F C K N R R H L E E T K  
 \* R \* \* N K I Y S V K I E D I \* K K L K  
 GTTAAAGATAATAGATAAAATCTATTCTGTAAAAATAGAAGACATTTAGAAGAACTAA  
 370 380 390 400 410 420

N L Y L \* M E L S E P I N L I F V I Y \*  
 I S I F R W S \* A S L L I L S S L F T K  
 S L S L D G V K R A Y \* S Y L R Y L L N  
 AATCTCTATCTTAGATGGAGTTAAGCGAGCCTATTAAATCTTATCTTCGTTATTTACTAA  
 430 440 450 460 470 480

T K \* Y L N C L V F K L I S I D N \* I I  
 L S N I \* I A W Y S S \* F Q \* I I K \* L  
 \* V I F K L L G I Q V N F N R \* L N N \*  
 ACTAAGTAATATTAAATTGCTGGTATTCAAGTTAATTCAATAGATAATTAAATAATT  
 490 500 510 520 530 540

N K Q N S A Y \* N L K K C Y Y Q V \* L V  
 I N R I R L I K I \* K N V I I K Y N \* S  
 \* T E F G L L K S E K M L L S S I S C  
 AATAAACAGAATTGGCTTATTAAATCTGAAAAATGTTATTCAAGTATAATTAGTC  
 550 560 570 580 590 600

S \* L L \* L F L L L T G I \* F C \* V E \*  
 A N C Y S S F F S L E F S F V E \* N K  
 L I A I A L S S S H W N L V L L S R I S  
 AGCTAATTGCTATAGCTTTCTTCTTCACTGGAATTAGTTTGTTGAGTAGAATAA  
 610 620 630 640 650 660

V L F P \* S I L \* F \* H I M F I M \* R L  
 Y Y F L N L F Y N F D I \* C L L C R D Y  
 I I S L I Y S I I L T Y N V Y Y V E I I  
 GTATTATTCCTTAATCTATTCTATAATTGACATATAATGTTATTATGTAGAGATTA  
 670 680 690 700 710 720

\* G \* V \* E S I M D F Y K \* L V \* H N L  
 R V R F R N L \* W I F T S N \* F N T I C  
 G L G L G I Y N G F L Q V T S L T Q F V  
 TAGGGTTAGGTTAGGAATCTATAATGGATTTACAGTAACAGTTAACACAAATTG  
 730 740 750 760 770 780  
 oligo 8F

L I S L F F S \* E F \* Y W V L Q G S I M  
 \* Y L Y F S L R N F N I G Y Y R V L S C  
 D I F I F L L G I L I L G I T G F Y H V  
 TTGATATCTTATTTCTTAGGAATTAAATATTGGTATTACAGGGTTCTATCATG  
 790 800 810 820 830 840

NADH dehydrogenase subunit 2

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L T K T I H E N L Q V F M I L N N I \* S  
 \* Q R G F T R T Y K S L \* F \* T T F R V  
D K D N S R E L T S L Y D F K Q H L E Y  
 TTGACAAAGACAATTACGAGAACTTACAAGTCTTATGATTTAACACATTTAGAGT  
 850 860 870 880 890 900

I Q F \* V F S Y Y L E V K F \* \* V H \* M  
S S S K S F L I I W K S N S S K F I E C  
P V L S L F L L F G S Q I L V S S L N V  
 ATCCAGTTCTAAGTCTTTCTTATTATTTGGAAAGTCAAATTCTAGTAAGTTCAATTGAATG  
 910 920 930 940 950 960

\* L L S I Y L \* N Y K V S L F I S C H L  
D Y F L F I F R I T K F L S L Y L V I F  
I T F Y L S L E L Q S F S L Y I L S S L  
 TGATTACTTTCTATTATTTAGAATTACAAAGTTCTCTCTTTATATCTTGTCACTT  
 970 980 990 1000 1010 1020

\* D P L N K V \* N I F Y \* G L Y L L A L  
 K I L \* T R F K I F F I R G S I F L L Y  
R S S K Q G L K Y F L L G A L S S C F I  
 TAAGATCCTCTAAACAAGGTTAAATATTAGGGCTATCTTGTCACTT  
 1030 1040 1050 1060 1070 1080

F Y \* D L V W C T V I Q E \* H L \* N L \*  
S I R I W F G V Q L Y R N N I F R I S S  
L L G F G L V Y S Y T G I T S L E S L A  
 TTCTATTAGGATTGGTTGGTGTACAGTTATACAGGAATAACATCTTGAATCTCTAG  
 1090 1100 1110 1120 1130 1140

R Y L V R L I \* I F I C R L V Y \* F V Y  
 D I \* \* G \* S K Y L Y A D \* F I N L C I  
I F S K V N L N I Y M Q I S L L I C V L  
 CGATATTTAGTAAGGTTAATCTAAATATTATATGCAGATTAGTTATTAAATTGTGTAT  
 1150 1160 1170 1180 1190 1200

\* E F S L K \* G \* Y L S I N G Q S M F M  
R N S L \* N R D S T F P S M G N R C L \*  
G I L F K I G I V P F H Q W A I D V Y D  
 TAGGAATTCTCTTAAATAGGGATAGTACCTTCCATCAATGGGCAATCGATGTTATG  
 1210 1220 1230 1240 1250 1260  
 oligo 5R

M E Y Q Q \* \* R P G \* Q L \* Q K Y L Y \*  
W S T N N N N D L V N N F N K N I F I N  
G V P T I I T T W L T T L T K I S L L I  
ATGGAGTACCAACAAATAATAACGACCTGGTTAACAACTTAAACAAAAATATCTTTATTAA  
 1270 1280 1290 1300 1310 1320  
 oligo 5R

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Y S \* W S L F I I I H Q K I G Q Q Y \* C  
 I L N G V Y L S S F I R K L D N N I N V  
 F L M E F I Y H H S S E N W T T I L M L  
 TATTCTTAATGGAGTTTATTCATCATTATCAGAAAATTGGACAAACAATATTAATGT  
 1330 1340 1350 1360 1370 1380

Y Y Q C Y L \* \* W G L S W D Y L N P V L  
 I I S V I C D S G V S P G I I S I P Y \*  
 L S V L S V I V G S L L G L S Q S R I K  
 TATTATCAGTGTATCTGTGATAGTGGGGTCTCTCCTGGGATTATCTCAATCCCGTATTA  
 1390 1400 1410 1420 1430 1440  
 oligo 5F(R/C)

N D Y \* S I A W \* V M \* D F \* C Y P Y Q  
 T I I N L \* H G K S C R I F N A I L I N  
 R L I Y S M V S H V G F L M L S I S I  
 AACGATTATTAATCTATAGCATGGTAAGTCATGTAGGATTTAATGCTATCCTTATCAA  
 1450 1460 1470 1480 1490 1500

\* \* Q R N L \* K H S Y F I \* Y N I V \* Q  
 N D R E I F R S I L I L F S T I \* Y N K  
 M T E K S L E A F L F Y L V Q Y S I T N  
 TAATGACAGAGAAATCTTAAAGCATTCTTATTTATTTAGTACAATATAAGTATAACAA  
 1510 1520 1530 1540 1550 1560

I \* M S F \* F \* L L W D I S T K I Q I V  
 F K C L F N S D C Y G I F L Q K S R \* \*  
 L N V F L I L I A M G Y F Y K N P D S E  
 ATTTAAATGTCTTTAAATTCTGATTGCTATGGATATTCACAAAAATCCAGATAGTG  
 1570 1580 1590 1600 1610 1620

K I L Q \* F I S I V \* E V W \* E S S P Y  
 R F S N N L Y Q \* F K R F G E S P A L I  
 D S P I I Y I N S L R G L V P V Q P L L  
 AAGATTCTCCAATAATTATATCAATAGTTAAGAGGTTGGTGAGAGTCCAGCCCTTAT  
 1630 1640 1650 1660 1670 1680

Y L F V \* P S L Y Y L W G E Y R L L \* D  
 I Y L F S H L F T I S G G N T A F Y R I  
 S I C L A I S L L S L G G I P P F I G F  
 TATCTATTGTTAGCCATCTTACTATCTCTGGGGGGAAATACCGCCTTTATAGGAT  
 1690 1700 1710 1720 1730 1740

F L V S \* I F Y I V R \* L K D I Y L Y L  
 F W \* V K Y S I \* Y D N S R I F I Y I Y  
 F G K L N I L Y S T I T Q G Y L F I S I  
 TTTTGGTAAGTTAAATATTCTATATAGTACGATAACTCAAGGATATTTATATCTA  
 1750 1760 1770 1780 1790 1800

NADH dehydrogenase subunit 2

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Y F L S \* R V F \* V \* V I I \* K W Y N C  
 T S C L S E C F K Y K L L F K S G T I V  
L L V L A S V L S I S Y Y L K V V Q L L  
 TACTTCTTGTCTTAGCGAGTGTAAAGTATAAGTTATTATTAAGTGGTACAATTGT  
 1810 1820 1830 1840 1850 1860

Y L W E S L L \* V L E I Y R F L H I \* V  
 I C G R V F F K F \* K Y T D F Y I F K Y  
F V G E S S L S F R N I Q I S T Y L S T  
 TATTGTGGGAGAGTCTTCTTAAGTTAGAAAATACAGATTCTACATATTAAAGTA  
 1870 1880 1890 1900 1910 1920

H \* S V F \* L \* \* \* Q C F \* L T L I L Y  
 I N R C S N F N D S N V F S \* P \* F Y I  
L I G V L T L M I A M F L V N P D F I L  
 CATTAATCGGTGTTCTAACTTTAATGATAGCAATGTTAGTTAACCTGATTATAT  
 1930 1940 1950 1960 1970 1980  
 oligo4R

Y N \* \* I \* Q F V N I L F Y N \* \* S M Q  
 T I N K Y N N L \* I F Y S I T S N P C K  
Q L I N I T I C K Y F I L \* L V I H A N  
 TACAATTAAATAAAATATAACAATTGTAAATTTATTCATAACTAGTAATCCATGCAA  
 1990 2000 2010 2020 2030 2040

I L V I V T V A I S L S L I I L N V L L  
Y \* \* \* L Q \* L F H Y H \* \* Y \* M F Y \*  
 I S N S Y S S Y F I I I N N I K C F I S  
 ATATTAGTAATAGTTACAGTAGCTATTCTATTATAATATTAAATGTTTATTAA  
 2050 2060 2070 2080 2090 2100

A K T S P T L E K V S P F E C G F S S F  
L R P L G H \* R K F L P L N V D L V L F  
 \* D L S N I R E S F S L \* M W I \* F F S  
 GCTAAGACCTCTCCAACATTAGAGAAAAGTTCTCCCTTGAATGTGGATTAGTTCTTT  
 2110 2120 2130 2140 2150 2160  
 oligo4F(R/C)

H Q T R S P F N I Y Y Y L I G L L F L I  
I K R E V L L T F I I I \* \* V Y Y F \* S  
 S N A K S F \* H L L L F N R F I I S N L  
 CATCAAAACGCGAAGTCCTTTAACATTATTATTAAATAGGTTATTATTCATAATC  
 2170 2180 2190 2200 2210 2220

NADH dehydrogenase subunit 2

NADH dehydrogenase subunit 3

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F D L E I L L I Y P Y A L F T T T Y G F  
 L I \* K F Y \* F I P M L Y L Q Q P M D F  
 \* F R N F I N L S L C F I Y N N L W I L  
 T T G A T T A G A A A T T T A T T A T T C C T A T G C T T A T T A C A A C A A C C T A T G G A T T T  
 2230 2240 2250 2260 2270 2280

Y I F N I F L I F L T I G F I Y E F G K  
 I Y L I Y F \* S F \* Q \* V L Y T N L A R  
 Y I \* Y I F N L F N N R F Y I R I W Q G  
 T A T A T T A A T A T A T T T A A T C T T T A C A A T A G G T T A T A C G A A T T G G C A A G  
 2290 2300 2310 2320 2330 2340

G V L K F K T H E \* Y Y T \* R C T H S L  
 E F \* N L R P M N N I I H N D A P T P W  
 S F K I \* D P \* I I L Y I T M H P L L G  
 G G A G T T T A A A T T A A G A C C C A T G A A T A T T A C A T A A C G A T G C A C C C A C T C C T G  
 2350 2360 2370 2380 2390 2400

G Y I F P R W S E S R L \* W Y S R I T \*  
 G I Y F Q D G A S P V Y D G I V E L H D  
 V Y I S K M E R V P S M M V \* \* N Y M T  
 G G G T A T A T T C C A A G A T G G A G C G A G T C C C G T C T A T G A T G G T A T A G T A G A A T T A C A T G A  
 2410 2420 2430 2440 2450 2460

P S S F L L T N S I S R S F L D S V L Y  
 Q V L F Y L L I V L V G V S W I L F S T  
 K F F F T Y \* \* Y \* \* E F L G F C S L Q  
 C C A A G T T C T T T T A C T T A C T A T A G T A T T A G G A G T T C T G G A T T C T G T T C T C A C  
 2470 2480 2490 2500 2510 2520

N F T I Q R F R D R P \* I S \* S \* Y N Y  
 I L R F R G S G I V H K Y H N H S T T I  
 F Y D S E V Q G S S I N I I I I V Q L \*  
 A A T T T A C G A T T C A G A G G T T C A G G G A T C G T C C A T A A A T A T C A T A A T C A T A G T A C A A C T A T  
 2530 2540 2550 2560 2570 2580

R I C L D S E S S S T F T N S H C F S K F  
 E F V W T V S P A L L I A I A F P S F  
 N L F G Q \* V Q H F Y \* \* P L L F Q V S  
 A G A A T T T G T T G G A C A G T G A G T C C A G C A C T T T A C T A A T A G C C A T T G C T T T C C A A G T T T  
 2590 2600 2610 2620 2630 2640  
 oligo 3F

Q I I . V F N G \* S D R S I H N N \* S D P  
 K L L Y L M D E V I D P S I T I K A I G  
 N Y C I \* W M K \* S I H P \* Q L K R \* V  
 C A A A T T A T T G T A T T A T G G A T G A A G T G A T C G A T C C A T C C A T A A C A A T T A A A G C G A T A G G  
 2650 2660 2670 2680 2690 2700

NADH dehydrogenase subunit 3

Cytochrome oxidase subunit II

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S S M V L V L \* I L R L Y R Q R G S I Y  
H Q W Y W S Y E Y S D Y T D K E G Q S I  
I N G I G P M N T P I I Q T K R V N L \*  
TCATCAATGGTATTGGTCTATGAATACTCCGATTATACAGACAAAGAGGGTCAATCTAT  
2710 2720 2730 2740 2750 2760

R I \* F L Y V T H R R S \* G G S I K T I  
E F D S Y M L P T E D L E E G Q L R Q L  
N L I L I C Y P Q K I L R R V N x D N \*  
AGAATTGATTCTTATATGTTACCCACAGAAGATCTTGAGGAGGGTCAATTAAAGACAATT  
2770 2780 2790 2800 2810 2820

R G \* \* P S L S S S E Y S S S I Y Y Y C  
E V D N R V L V P V N T P L R F I I T A  
R L I T E S \* F Q \* I L L F C L L L L L  
AGAGGTTGATAACCGAGTCTTAGTCCAGTGAATACTCCTCTTCGATTATTACTGC  
2830 2840 2850 2860 2870 2880

Y R C F T \* F C G S F F R N Q S G C E S  
T D V L H D F A V P S L G I K V D A S P  
Q M F Y M I L R F L L \* E S K W M R V Q  
TACAGATGTTTACATGATTTGCGGTCTCTTTAGGAATCAAAGTGGATGCGAGTC  
2890 2900 2910 2920 2930 2940

R S I K S S I N I C T T \* R S V L W S M  
G R L N Q V S T Y V Q R E G V Y Y G Q C  
V D \* I K Y Q H M Y N V K E C I M V N V  
AGGTGATTAAATCAAGTATCAACATATGTACAACGTGAAGGAGTGTATTATGGTCAATG  
2950 2960 2970 2980 2990 3000

\* \* T M W C I T \* \* Y A D C H R G S L F  
S E L C G V L H S S M P I V I E A V S L  
V N Y V V Y Y I V V C R L S S R Q S L \*  
TAGTGAACATGTGGTGTATTACATAGTAGTATGCCGATTGTATCGAGGCAGTCTCTT  
3010 3020 3030 3040 3050 3060

R K I F I L V G \* S I I H H S S I Y S P  
E K F L S W L D N Q \* S I I L P F T L R  
K N F Y L G W I I N N P S F F H L L S V  
AGAAAAATTTTATCTTGGTGGATAATCAAATAATCCATCATTCTCCATTACTCTCCG  
3070 3080 3090 3100 3110 3120  
oligo 3R (R/C)

Cytochrome oxidase subunit II

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\* A K K I D W N R I I Y F \* T D S S \* K  
 E Q R K \* I G I E L S I F K R I V H E S  
 S K E N R L E \* N Y L F L N G \* F M K V  
 TGAGCAAAGAAAATAGATTGGAATAGAATTATCTATTTAAACGGATAGTTCATGAAAG  
 3130 3140 3150 3160 3170 3180

S E F N V S S E \* T L S R G I T H A N R  
 Q S L M L A Q N E R Y L E A L H M Q I V  
 R V \* C \* L R M N A I \* R H Y T C K S S  
 TCAGAGTTAATGTTAGCTAGAACGCTATCTAGAGGCATTACACATGCAAATCGT  
 3190 3200 3210 3220 3230 3240

Q G W F T I P T V Y R \* V \* I G I Y P L  
 R G G L P F L R C T G E Y K \* E S T H \*  
 G V V Y H S Y G V Q V S I N R N L F I N  
 CAGGGGTGGTTACCATTCCTACGGTGTACAGGTGAGTATAAATAGGAATCTACCCATTA  
 3250 3260 3270 3280 3290 3300

T F \* E L V D E P I L G K V V G G T K A  
 H S K S \* W M S L S W G R \* L V G Q K L  
 I L R V S G \* A Y L G E G S W W D K S L  
 ACATTCTAAGAGTTAGTGGATGAGCCTATCTGGGGAAAGGTAGTTGGGACAAAAGCT  
 3310 3320 3330 3340 3350 3360

Y Q A R E P \* S M F E R T S D H I G S E  
 T K P E N P S Q C L K E P L T T L A L K  
 P S Q R T L V N V \* K N L \* P H W L \* N  
 TACCAAGCCAGAGAACCCCTAGTCATGTTGAAAGAACCTCTGACCACATTGGCTCTGAA  
 3370 3380 3390 3400 3410 3420

T I A K I L Y Q G V Q Q \* G I L V N D R  
 Q \* P R F S T R E S S S E E Y W S M I A  
 N S Q D S L P G S P A V R N I G Q \* S Q  
 ACAATAGCCAAGATTCTCTACCAAGGGAGTCAGCAGTGAGGAATATTGGTCAATGATCGC  
 3430 3440 3450 3460 3470 3480

K I E P A I \* K N F Y I L N K E R M M T  
 R L N Q P S R R I F I F \* T K R G \* \* R  
 D \* T S H L E E F L Y S K C R E D D D V  
 AAGATTGAACCAGCCATCTAGAAGAATTCTAAACAAAGAGAGGATGATGAGC  
 3490 3500 3510 3520 3530 3540

L S L L Q S R P N L V P A V A V I R V R  
 Y L C Y S L D P I S C Q Q S R \* Y E \* G  
 I F V T V S T Q S R A S S R G N T S E A  
 TTATCTTGTTACAGTCTCGACCCAAATCTCGTGCAGCAGTCGCGGTAAACGAGTGAGG  
 3550 3560 3570 3580 3590 3600

Small subunit rRNA

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Q A L F I I T R S K G \* V G G Y E L I N  
 K R Y S S L L G L K G E \* V V M N L L I  
 S V I H H Y \* V \* R V S R W L \* T Y \* L  
 CAAGCGTTATTCTATTAGGTCTAAAGGGTAGGTGGTTATGAACTTATTAAT  
 3610 3620 3630 3640 3650 3660

\* L E S N R R I K N F G S R D E I R \* Y  
 N \* S R I E E \* R I L G V E M K S D D T  
 T R V E S K N K E F W E \* R \* N P M I P  
 TAACTAGAGTCGAATCGAAGAATAAGAATTGGGAGTAGAGATGAAATCGATGATAC  
 3670 3680 3690 3700 3710 3720

P K D C S W R K H Y S N Y R L T L R Y E  
 Q R T A H G E S I I L I I D \* H \* G T K  
 K G L L M A K A L F \* L S T D T E V R K  
CGAAAGGACTGCTCATGGCGAAAGCATTATTCTAATTATCGACTGACACTGAGGTACGAA  
 3730 3740 3750 3760 3770 3780  
 oligo 7R(R/C)

S I R R R K D \* I P L \* F M L \* T M N A  
 A \* G G A R I R Y P C S L C C K R \* M L  
 H K E A Q G L D T L V V Y A V . N D E C \*  
 AGCATAAGGAGGCGCAAGGATTAGATAACCCTGTAGTTATGCTGTAAACGATGAATGCT  
 3790 3800 3810 3820 3830 3840

R N \* N T L F \* F L W \* R L \* A F H L R  
 E I R I L Y F S F C G E D F K H S T \* E  
 K L E Y S I L V S V V K T L S I P P E K  
 AGAAATTAGAATACTCTATTTAGTTCTGTGGTAGACTTTAACGATTCCACCTGAGA  
 3850 3860 3870 3880 3890 3900

S T V A R L K L K T L D G H R D Q Q \* S  
 V L S Q G \* N S K H \* T V T E I S S E A  
 Y C R K A E T Q N I R R S Q R S A V K H  
 AGTACTGTCGCAAGGCTGAAACATTAGACGGTCACAGAGATCAGCAGTGAAGC  
 3910 3920 3930 3940 3950 3960

M L F N S I T H D K S Y H S L Y N K Y F  
 C C L I R \* P T T N L T T P C I I N I F  
 V V \* F D N P R Q I L P L L V \* \* I F S  
 ATGTTGTTAACCGACAAACTCTTACCACTCCTTGTATAATAATATTTT  
 3970 3980 3990 4000 4010 4020

P \* G I V L T L R N F N \* I I T . Y I L M  
 L K G L F \* L \* G T L I K \* \* H I Y L \*  
 L R D C F D F E E L \* L N N N I Y T Y D  
 CCTTAAGGGATTGTTTGACTTGAGGAACCTTAATAAACATATATACTTATG  
 4030 4040 4050 4060 4070 4080

Small subunit rRNA

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I S L \* T Y L Y I F V I R H L N L Y \* \*  
 \* V F K L T Y I Y L \* Y V I \* T Y I N N  
 K S L N L L I Y I C N T S S K L I L I I  
 ATAAGTCTTAAACTTACTTATATATATTGTAATACGTCATCTAAACTTATATTAAATAA  
 4090 4100 4110 4120 4130 4140

\* F N S Y Y \* I D D I I V V N V M D S N  
 N S I V I I E S M T \* L L L M \* W I V I  
 I Q \* L L L N R \* H N C C \* C D G \* \* \*  
 TAATTCAATAGTTATTGAATCGATGACATAATTGTTGTTAATGTGATGGATAGTAAT  
 4150 4160 4170 4180 4190 4200

N N \* I L L W V \* V T L V I I T N Y Y H  
 I I K Y C Y G C E \* H \* \* \* L I I T I  
 \* L N I V M G V S N I S N N N \* L L P L  
 AATAATTAAATATTGTTATGGGTGTGAGTAACATTAGTAATAAACTAATTATTACCAT  
 4210 4220 4230 4240 4250 4260

Y K P Q \* \* \* L I K \* L N G M N D \* A I  
 I N H S N N N \* L N N \* M E \* M I K Q L  
 \* T T V I I I D \* I I K W N E \* L S N \*  
 TATAAAACCACAGTAATAAAATAATTGATTAAATAATTAAATGGAATGAATGATTAAGCAATT  
 4270 4280 4290 4300 4310 4320

S N S L I F I T G V A W L S L V R V V K  
 V I H \* Y L L Q V L H G C L \* F V L \* N  
 \* F I N I Y Y R C C M A V F S S C C E M  
 AGTAATTCAATTAATTATTACAGGTGTTGCATGGCTGTCTTAGTTCTGTGTTGAAA  
 4330 4340 4350 4360 4370 4380  
 oligo 2R

C \* V N S E N E R N P Y L Y L K L Y K E  
 V R L I P K T N A I L I F I \* N Y I K R  
 L G \* F R K R T Q S L S L F K T I \* R G  
 TGTTAGGTTAATTCCGAAAACGAAACGCAATCCTTATCTTATTAAACTATATAAGAG  
 4390 4400 4410 4420 4430 4440

V S F H K K E \* L G \* R G V L M T L M E  
 Y L S I R R N N \* G E D K S S \* P L W S  
 I F P \* E G I I R V K T S P H O P Y G V  
 GTATCTTCCATAAGAAGGAATAATTAGGGTGAAGACAAAGTCCTCATGACCCCTATGGAG  
 4450 4460 4470 4480 4490 4500

W A T D V P Q I F L Q R E A K M K V \* A  
 G L Q T C H K Y F Y K G K Q R \* K S E L  
 G Y R R A T N I S T K G S K D E S L S \*  
 TGGGCTACAGACGTGCCACAAATATTCTACAAAGGGAGCAGAAAGATGAAAGTCTGAGCT  
 4510 4520 4530 4540 4550 4560

Small subunit rRNA

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N P Q K K \* K Y G \* E S G T R F F E E G  
 I L K R N K S T D K N L E L D S L K K E  
 S S K E I K V R I R I W N S I L \* R R N  
 AATCCTCAAAAGAAAATAAAAGTACGGATAAGAATCTGGAACTCGATTCTTGAAGAAGGA  
 4570 4580 4590 4600 4610 4620

oligo 2F(R/C)

I A S N R S S S R N G E T N I C D V L T  
 L L V I V H H Q G T V K R T S V M Y \* L  
 C \* \* S F I I K E R \* N E H L \* C T N Y  
 ATTGCTAGTAATCGTTCATCATCAAGGAACGGTGAACATCTGTGATGTACTAATC  
 4630 4640 4650 4660 4670 4680

T R Q A R K S L R S I K L I E F N F \* R  
 L V K R E N H \* E V S S \* L N L I S K E  
 S S S A K I I K K Y G V D \* I \* F L K S  
 ACTCGTCAAGCGCGGAAAATCATTAAGAAGTATCAAGTTGATTGAATTAAATTCTAAAGA  
 4690 4700 4710 4720 4730 4740

oligo 1R

V K E F N I C R I K G F Q R L F P R N L  
 L K N L T S V E S K D F S V Y F L E I C  
 \* R I \* H L \* N Q R I S A S I S \* K F V  
 GTTAAAGAATTAAACATCTGTAGAATCAAAGGATTCAAGCGTCTATTCTAGAAATTG  
 4750 4760 4770 4780 4790 4800

C \* V E I R \* L \* G N L \* L K D \* I T H  
 A K S K \* G S C R G T C S \* K I K \* P I  
 L S R N K V A V G E P V A E F L N N P \*  
 TGCTAAGTCGAAATAAGGTAGCTGTAGGGAACCTGTAGCTGAAAGATTAAACCCAT  
 4810 4820 4830 4840 4850 4860

N P T S F L K R R I L I V V P R I G C K  
 T P P H F L R E E S \* \* W S Q G \* A V N  
 P H L I S \* E K N L D S G P K D R L \* T  
 AACCCCCACCTCATTTCTTAAGAGAAGAACTTGTAGTAGTGGTCCCAAAGGATAGGCTGTAAA  
 4870 4880 4890 4900 4910 4920

P I S F N T C E G S T P S L L I L R L T  
 L \* V L I L V R V R L P L F S Y F D \* L  
 Y K F \* Y L \* G F D S L S S H T S I D S  
 CCTATAAGTTTAATACTTGTGAGGGTTCGACTCCCTCTCTCTCATACTTCGATTGACT  
 4930 4940 4950 4960 4970 4980

L S E I L R L I F L I L R L S F S H A S  
 S L S Y F D L S F S Y F D C L F L M L Q  
 L S H T S T Y L S H T S T V F F S C F K  
 CTCTCTCTCATACTTCGACTTATCTTCTCATACTTCGACTGTCTTTCTCATGCTTCA  
 4990 5000 5010 5020 5030 5040

oligo 9F

Small subunit rRNA

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S D S S H L F D L L Y S G \* F D Y L F L  
 V T L L I Y S I Y F I P N S L I I Y S Y  
 \* L F S F I R F T L F P I V \* L F I P T  
AGTGACTCTTCTCATTTATTCGATTTACTTATTC  
 5050 5060 5070 5080 5090 5100

L V Q \* L C S T L I N L I N S I L M F \*  
 S F N D F V L L L \* I \* S I L F L C F E  
 R S M T L F Y S Y K F N Q F Y S Y V L N  
CTCGTTCAATGACTTTGTTCTACTCTTATAAAATTAA  
 5110 5120 5130 5140 5150 5160

I V T I A \* W \* S K A L L M L Q Q K S D  
 \* L L \* L S G K A K H C \* C F N R S P I  
 S Y Y S L V V K Q S T V N A S T E V R F  
ATAGTTACTATAGCTTAGTGGTAAAGCAAAGCACTGTTAATGCTTAA  
 5170 5180 5190 5200 5210 5220

S S \* \* R M F S L E N S T S C L S I L L  
 L L S N E C L V \* K T Q Q V A \* A F Y \*  
 F L V T N V \* F R K L N K L L E H S I S  
TCTTCTTAGTAACGAATGTTAGTTAGAAA  
 5230 5240 5250 5260 5270 5280

A I L V V T S K N P V L S L L Y L I G L  
L Y \* W \* L L R I Q F F L Y Y I \* L D Y  
 Y I S G N F \* E S S S F F I I F D W I I  
GCTATATTAGTGGTAAC  
 5290 5300 5310 5320 5330 5340  
 oligo IF(R/C)

F I D I G V Y L I S L Q L T Y L G L S Y  
L \* I \* V C I \* Y L F S \* L T \* G Y H I  
 Y R Y R C V F D I S S V N L L R V I I Y  
TTTATAGATATAGGTGTGTTAGATATCTCTTCAGTTAA  
 5350 5360 5370 5380 5390 5400

I T V Y V G A I A M L F I F V I M M L N  
 \* L Y M L E L L Q C C L S L \* L \* C \* I  
 N C I C W S Y C N V V Y L C N Y D V K Y  
ATAACTGTATATGTTGGAGCTATTGCAATGTTGTTATCTTGTAA  
 5410 5420 5430 5440 5450 5460

I Q V V E S K K K T  
S K L \* N L K R R C  
 P S C R I \* K E D K  
ATCCAAGTTGTTAGAATCTAAAAAGAACAAA  
 5470 5480 5490

I P L G L  
V Y P \* V Y  
 Y T L R F I  
STATACCCCTTAGGTTA  
 5510 5520

**SUBSTITUTE SHEET**

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F L G S S L V F T L Y L S L P R E I I M  
F \* D L A \* Y L H F I Y L Y Q E K \* \* W  
 F R I \* L S I Y T L F I S T K R N N N G  
 TTTTTAGGATCTAGCTTAGTATTACACTTATTATCTACCAAGAGAAAATAATAATG  
 5530 5540 5550 5560 5570 5580

E K Y S H L F S Y F S W E N R I L N P S  
R N I L I S S H T S L G K I E F \* I L Q  
 E I F S S L L I L L L G K \* N F K S F S  
 GAGAAAATATTCTCATCTCTCATACTTCTCTGGGAAAAATAGAATTAAATCCTTC  
 5590 5600 5610 5620 5630 5640

V V E I L G K V L Y T D Y S L W L L I  
S \* K Y \* V K Y F I Q T I L S G Y C \* \*  
 R R N T R \* S T L Y R L F S L V I V N K  
 GTCGTAGAAATACTAGGTAAGTACTTATACAGACTATTCTCTGGTTATTGTTAATA  
 5650 5660 5670 5680 5690 5700

S L I L V L A I V G A I S I A A H K E \*  
V \* Y W C \* L L L E P F L \* Q L T K N K  
 S N I G A S Y C W S H F Y S S S Q R I S  
 AGTCTAATATTGGTGCTAGCTATTGGAGCCATTCTATAGCAGCTCACAAAGAATAA  
 5710 5720 5730 5740 5750 5760

V N I I H Q L M L F E V L V L I V S V L  
L I L S I N \* C Y L K Y \* Y \* \* Y P Y C  
 \* Y Y P S I N V I \* S I S I N S I R T V  
 GTTAATATTATCCATCAATTAAATGTTATTGAAGTATTAGTATTAAATAGTATCCGTACTG  
 5770 5780 5790 5800 5810 5820

L S V A Y L T L A E R K V M G S M Q R R  
\* V L L I \* P \* Q R E K \* W D L C K D V  
 K C C L S N L S R E K S D G I Y A K T F  
 TTAAGTGTGCTTATCTAACCTTAGCAGAGAGAAAAGTGTGGATCTATGCAAAGACGT  
 5830 5840 5850 5860 5870 5880

L G P N A V G Y Y G L L Q P F A D A L K  
\* D R M P \* D I M V Y Y N P L Q M P \* N  
 R T E C R R I L W F I T T L C R C L K I  
 TTAGGACCGAATGCCGTAGGATATTATGGTTATTACAACCCTTGCAGATGCCTTAAAA  
 5890 5900 5910 5920 5930 5940

L I V K E T I I P S Q A N K I L F F L G  
\* \* \* K K Q \* Y L L K P I K S Y S F \* V  
 D S E R N N N T F S S Q \* N L I L S R S  
 TTGATAGTGAAGAAAACAATAATACCTTCTCAAGCCAATAAAAATCTTATTCAGGT  
 5950 5960 5970 5980 5990 6000

NADH dehydrogenase 6

NADH dehydrogenase 1

SUBSTITUTE SHEET

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P M I A L V F A L L G W G L I P Y G P G  
L \* L L \* S L P C \* D G V \* Y L M G L G  
 Y D C F S L C L V R M G S N T L W A W G  
 CCTATGATTGCTTAGCTTTGCCTGTTAGGAATGGGGTCTAATACCTTATGGGCCTGGG  
 6010 6020 6030 6040 6050 6060

oligo 6R

A T I C D F E L G V L Y S L A I S S V G  
Q G S V I L N \* E F S I V \* L F L L \* G  
 N N L \* F \* I R S S L \* F S Y F F C R G  
 GCAACAACTCTGTGATTTGAATTAGGAGTCTCTATAGTTAGCTATTCCTCTGTAGGG  
 6070 6080 6090 6100 6110 6120

V Y G I L I G G W S S N S K Y P L V G S  
F T E S \* \* G V G H P I P N I L \* \* V L  
 L R N L N R G L V I Q F Q I S F S R F S  
 GTTTACGGAATCTTAATAGGGGGTTGGTCATCCAATTCCAAATATCCTTAGTAGGTTCT  
 6130 6140 6150 6160 6170 6180

oligo 6F(R/C)

L R S T A Q L I S Y E L V L T S I V F I  
\* G V Q L N \* L V M N \* F L L R L Y S S  
 K E Y S S I N \* L \* T S S Y F D C I H H  
 CTAAGGAGTACAGCTCAATTAAATTAGTTATGAACTAGTTCTTACTTCAATTCTTCTATT  
 6190 6200 6210 6220 6230 6240

I V F F S G T L N W T Q L V E A Q H S I  
L S S S L E L L I G L N W S K L N I L F  
 C L L L W N S \* L D S I G R S S T F Y L  
 ATTGTCTTCTCTCTGGAACTCTTAATTGGACTCAATTGGTCGAAGCTAACATTCTATT  
 6250 6260 6270 6280 6290 6300

W Y C I P L L P L F V M Y F I G A L A E  
G I A Y L F Y H F L S C I S L E L \* L K  
 V L H T S S T T F C H V F H W S F S \* N  
 TGGTATTGCATACCTCTTACCACTTTTGTCATGTATTCATTGGAGCTTACGTGAA  
 6310 6320 6330 6340 6350 6360

T N R A P F D L P E A E S E L V A G F M  
Q I E L L L I Y P K R S L N \* L Q D L \*  
 K S S S F \* F T R S G V \* I S C R I Y D  
 ACAAAATCGAGCTCCTTTGATTTACCGAAGCGGGAGTCTGAATTAGTTGCAGGATTATG  
 6370 6380 6390 6400 6410 6420

T E Y S A A I F V Y Y F L A E Y G N I L  
Q N I P Q L Y S Y I T S \* Q N T G I F F  
 R I F R S Y I R I L L L S R I R E Y S F  
 ACAGAAATATTCCGCAGCTATATTCTGATATTACTTCTTAGCAGAAATACGGAAATATTCTT  
 6430 6440 6450 6460 6470 6480

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NADH dehydrogenase 1

L I S T L S V I F F L G G Y L L P F E G  
 \* S Q H Y Q \* S S S W E V I Y Y L S K V  
 N L N I I S D L L L G R L F I T F R R L  
 TTAATCTAACATTATCAGTGAATCTCTGGGAGGTTATTACCTTCGAAGGT  
 6490 6500 6510 6520 6530 6540

C L Q L V G I G L O S I T G Y R V P I L  
 V Y N L L E L V Y S L L A I E Y L F Y  
 S T T C W N W F T V Y Y W L \* S T Y F I  
 TGTCTACAACTTGTGGAATTGGTTACAGTCTATTACTGGCTATAGAGTACCTATTTA  
 6550 6560 6570 6580 6590 6600

F L T S S V T E G I F Y G L S L G I K V  
 S \* L L Q L Q K E S F M V F P \* V L K Y  
 L N F F S Y R R N L L W S F P R Y \* S I  
 TTCTTAACCTCTTCAGTTACAGAAGGAATCTTATGGTCTTCCCTAGGTATTAAAGTA  
 6610 6620 6630 6640 6650 6660

S L L I F L F I W V R A S F P R I R Y D  
 L Y \* Y F Y L Y G L E L L S H E \* D M I  
 F I N I F I Y M G \* S F F P T N K I \* S  
 TCTTTATTAATATTTATTTATGGTTAGAGCTTCTTCCACGAATAAGATATGAT  
 6670 6680 6690 6700 6710 6720

H I F Q R T L D H K G I I S D I P K D I  
 I Y S K G H \* I I R A L Y Q I F Q R T L  
 Y I P K D I R S \* G H Y I R Y ' S K G H \*  
 CATATATTCCAAAGGACATTAGATCATAAGGGCATTATATCAGATATTCCAAAGGACATT  
 6730 6740 6750 6760 6770 6780

R  
D  
I  
AGATC

## INTERNATIONAL SEARCH REPORT

International Appl. No PCT/GB 91/00869

## I. CLASSIFICATION

According to International Patent Classification (IPC) or to both National Classification and/or C  
 Int.C1.5 C 12 Q 1/68 C 12 P 19/34 C 07 H 21/04  
 C 07 K 15/02 // C 12 N 15/11

all)6

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols		
Int.C1.5	C 12 Q C 07 K	C 12 P A 61 K	C 12 N

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0327390 (ISIS INNOVATION LTD) 9 August 1989, see figures 2,3, which correspond to figure 3 of application, nucleotides 1-89,90-539 respectively (cited in the application)	2
Y	---	4,5,6,9
P,X	THE LANCET, vol. 336, no. 8713, August 1990, A.E. WAKEFIELD et al.: "Detection of Pneumocystis carinii with DNA amplification", pages 451-453, see the whole article	2,4-8
P,Y	WO,A,9013669 (XYTRONYX INC.) 15 November 1990, see the whole document ---	4,5,6,9

<sup>10</sup> Special categories of cited documents :<sup>10</sup>

- <sup>"A"</sup> document defining the general state of the art which is not considered to be of particular relevance
- <sup>"E"</sup> earlier document but published on or after the international filing date
- <sup>"L"</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- <sup>"O"</sup> document referring to an oral disclosure, use, exhibition or other means
- <sup>"P"</sup> document published prior to the international filing date but later than the priority date claimed

- <sup>"T"</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- <sup>"X"</sup> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- <sup>"Y"</sup> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- <sup>"&"</sup> document member of the same parent family.

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

09-09-1991

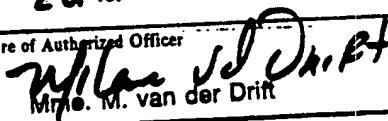
Date of Mailing of this International Search Report

28.10.91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer


  
Mr. M. van der Drift

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 111, no. 3, 3 July 1989, page 205, abstract no. 2066k, (Columbus, Ohio, US), A.E. WAKEFIELD et al.: "Cloning of DNA from <i>Pneumocystis carinii</i> ", & J. PROTOZOOL. 1989, 36(1), 55-75	
P, X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 45, no. 1, January 1991, Elsevier Science Publishers B.V. (Biomedical Division), K. SINCLAIR et al.: "Pneumocystis carinii organisms derived from rat and human hosts are genetically distinct", pages 183-184, see the whole article	1-8
P, X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 43, no. 1, November 1990, Elsevier Science Publishers B.V. (Biomedical Division), A.E. WAKEFIELD et al.: "Amplification of mitochondrial ribosomal RNA sequences from <i>Pneumocystis carinii</i> DNA rat and human origin", pages 69-76, see the whole article	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9100869  
SA 48160

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/10/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
EP-A- 0327390	09-08-89	JP-A-	2005899	10-01-90
WO-A- 9013669	15-11-90	AU-A-	5671190	29-11-90

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82